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菊科橐吾属植物的 DNA 条形码研究*

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摘要: DNA 条形码是一项利用短的、标准的 DNA 片段对物种进行快速、有效识别和鉴定的新技术。菊科橐吾属约 140 种, 是典型的高山植物, 种间杂交频繁, 形态变异复杂, 从形态学方面鉴定近缘种较为困难。本研究选取 4 个 DNA 核心条形码片段 (ITS, *mat*K, *psb*A-*trn*H 和 *rbc*L), 对橐吾属 35 种 144 个个体进行条形码研究。研究结果显示叶绿体基因 *mat*K, *psb*A-*trn*H 和 *rbc*L 在种内和种间变异都很小, 对橐吾属的物种鉴定率极低; ITS 在种间变异率相对较大, 物种鉴定率为 60%。而各片段联合后的物种鉴定率并未提高。

关键词: DNA 条形码; 橐吾属; 物种鉴别; ITS; *mat*K; *psb*A-*trn*H; *rbc*L
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Study on the DNA Barcoding of Genus *Ligularia* Cass. (Asteraceae)*

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Abstract: DNA barcoding is a new technology which can identify species rapidly based on short and standardized DNA sequences. *Ligularia*, a genus of Asteraceae with about 140 species, exhibits high morphological and ecological diversity, which makes the classification and species delimitation difficult, especially in the cases of closely related taxa. In this study, we tested four DNA core barcoding regions (ITS, *mat*K, *psb*A-*trn*H and *rbc*L) in 144 samples representing 35 species of *Ligularia*. The results revealed that the chloroplast regions (*mat*K, *psb*A-*trn*H and *rbc*L) have extremely low species identification rate due to low interspecific variation. Conversely, ITS sequence showed higher species identification rate (60%) and could discriminate the species which are difficult to identify. The combination of these four gene fragments did not improve the ability of species discrimination.

Key words: DNA barcoding; *Ligularia*; species identification; ITS; *mat*K; *psb*A-*trn*H; *rbc*L

DNA barcoding is a new technology which can identify species rapidly based on a short and standardized DNA sequence (Hebert *et al.*, 2003; Kress *et al.*, 2005; Hollingsworth, 2011; Schoch *et al.*, 2012). This technology has been widely used as a tool for species identification and discovery of cryptic species (Zemlak *et al.*, 2009; Liu *et al.*, 2011; Huemer *et al.*, 2014) since it was proposed (Hebert *et al.*, 2003). At present, the mitochondrial gene cytochrome *c* oxidase I (*CO*I) has been proven to be a universal and effective barcode for discriminating species in animals, such as birds (Hebert *et al.*, 2003, 2004), fishes (Ward *et al.*, 2005), insects (Linares *et al.*, 2009). However, gene *CO*I can not

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be applied in most of plant taxa because of the low substitution rate (Kress *et al.*, 2005). No DNA region universally suitable and meeting all of the necessary criteria has been found for all plants (Fazekas *et al.*, 2008; Hollingsworth *et al.*, 2011; Li *et al.*, 2011). Searching for DNA barcode in plants proved to be a more challenging task (Kress *et al.*, 2005; Chase *et al.*, 2007; Kress and Erickson, 2007; Lahaye *et al.*, 2008). Based on the previous research, *rbc*L + *mat*K has been suggested to be the core plant barcode by the Consortium for the Barcode of life (CBOL) Plant Working Group (PWG) (CBOL Plant Working Group, 2009). The China plant work group for the Barcode of life proposed that ITS/ITS2 should be incorporated into the core barcode for seed plants according to the study in 1 757 species of 75 families (Li *et al.*, 2011). And Chen *et al.* (2010) also found that the ITS2 performed well in the identification of medicinal plants. In addition, the plastid intergenic spacer region *psb*A-*trn*H was recommended as a candidate barcode (Hollingsworth *et al.*, 2011).

The four core barcodes (*rbc*L, *mat*K, *psb*A-*trn*H and ITS) have been proved to have highly universal primers and high species identification rate in some plant groups (Kress *et al.*, 2005, 2009; Kress and Erickson, 2007; Fazekas *et al.*, 2008; Lahaye *et al.*, 2008; Newmaster *et al.*, 2006; 2008; Hollingsworth *et al.*, 2009; Jiao and Shui; 2013; Liu *et al.*, 2013), but there are still debate about these DNA regions as barcodes. Of the four single-marker barcodes for plants, for example, ITS, as a standard barcode, is often questioned because of the potential fungal contamination and the presence of paralogous copies (Hollinsworth *et al.*, 2011). However, the research work including 6 286 individuals representing 1 757 species in 141 genera of 75 families revealed that ITS region performed the highest resolution in species discrimination of seed plants (Li *et al.*, 2011). Although *rbc*L showed good universality, its variation mainly existed above the species level and exhibited low species identification ability (Gonzalez *et al.*, 2009). *Mat*K showed high species identification ability in the study of Lahaye *et al.* (2008) and De Vere *et al.* (2012) with low primer generality, but it performed extremely low species identification rate in *Berberis* and *Ligustrum* (Roy *et al.*, 2010; Gu *et al.*, 2011). DNA segment *psb*A-*trn*H had high sequence variation in angiosperms (Kress *et al.*, 2005), and was considered perfect as a DNA barcode (Chase *et al.*, 2007; Roy *et al.*, 2010). However, its identification ability was poor in the medicinal plants *Paris* (Zhu *et al.*, 2010), and the sequence alignment was difficult because of the sequence structure variation (Kress *et al.*, 2005; Zhu *et al.*, 2010).

Ligularia Cass., belonging to the family Asteraceae, tribe Senecioneae, subtribe Tussilagininae, is a highly diversified genus. It includes six sections and about 140 species (Liu, 2011), and most of which are distributed in Asia with only two species in Europe (Liu, 1989; Liu *et al.*, 1994). Liu (1985, 1989) studied the Chinese species of *Ligularia* comprehensively and systematically, and 112 species were described, of which 67 species were distributed in Hengduan mountainous area with 61 species endemic to this area. The Hengduan mountains were considered to be the center of evolution and diversity for *Ligularia* (Liu *et al.*, 1994). The plants of this genus exhibit remarkably morphological variation in leaf shape and texture, indumenta and inflorescence among species. One species may present significant diversification in different populations. For example, *Ligularia sibirica*, the type species of this genus, has diversified leaf blade with ovate-cordate, triangular-cordate, reniform-cordate, or broadly cordate shape (Liu, 1989). This makes it difficult to identificate *Ligularia* species with traditional morphology-based methods, especially in the vegetative period of this taxa. DNA barcode technology can overcome this issue, and it only needs a small part of the organism tissue (e. g. a piece of leaf blade) to rapidly identify species. In this study, we tested the discriminatory power of four DNA fragments (ITS, *mat*K, *rbc*L and *psb*A-*trn*H) and evaluated the DNA barcoding per-

formance in species identification for the genus *Ligularia*.

1 Materials and methods

1.1 Experimental materials

A total of 144 individuals of 35 species from Yunnan, Sichuan, Qinghai and Gansu province were collected, and each species included at least two individuals (Table 1). The voucher specimens were deposited in Herbarium of Kunming Institute of Botany, CAS (KUN). Specimen identification was based on the classification system of Liu (1989).

1.2 Experimental methods

Total DNA was extracted from silica-gal dried leaf tissues using the CTAB method (Doyle and Doyle, 1987). Informations about the primers used in the present study are listed in Table 2. The PCR profiles for three chloroplast DNA fragments (*mat*K, *psb*A-*trn*H and *rbc*L) included an initial denaturation step at 94 ℃ for 5 min, followed by 30 cycles of 30 s at 94 ℃, 30 s at 53 ℃, 1 min at 72 ℃ and finished with an extension step of 7 min at 72 ℃. The PCR conditions for ITS consisted of an initial denaturation at 56 ℃ for 5 min, followed by 30 cycles of 30 s at 94 ℃, 30 s at 53 ℃, 1 min at 72 ℃ and finished with an extension step of 7 min at 72 ℃. The PCR reaction system was carried out in a total volume of 20 μL contained 13.1 μL of ultrapure water, 2.0 μL 10 × PCR buffer, 1.0 μL $MgCl_2$, 1.0 μL dNTP, 1.0 μL BSA, 0.3 μL Taq polymerase, 0.3 μL each primer, 1.5 μL template DNA (20−60 ng). The purified PCR products were run on an ABI 3730 automated sequencer, completed by sequencing company.

Table 1 The information of the samples

Species name	Sample ID	Locality	Latitude	Longitude	Atitude/m
L. hodgsonii	PG100806	Miyaluo, Li County, Sichuan	N31°38′18″	E102°49′10″	—
L. hodgsonii	PG100869	Guandi, Dangchang County, Gansu	N34°15′13″	E104°11′21″	2293
L. hodgsonii	PG100871	Wen County, Gansu	N33°05′21″	E104°45′51″	1462
L. stenoglossa	PG090978	Laojun Mountain, Yulong, Yunnan	N26°39′28″	E99°48′20″	3039
L. stenoglossa	PG100961	Cangshan, Dali, Yunnan	N25°40′48″	E100°05′41″	3760
L. duciformis	PG090905	Xiaozhongdian, Shangri-La, Yunnan	N27°38′32″	E99°47′48″	3607
L. duciformis	PG110810	Jiajinshan, Baoxing, Sichuan	N30°51′25″	E102°41′23″	3910
L. duciformis	PG110820	Damba, Sichaun	N30°32′06″	E101°35′55″	3750
L. duciformis	PG110873	Tianchi, Shangri-La, Yunnan	—	—	3890
L. nelumbifolia	PG090924	Xiaoxueshan, Shangri-La, Yunnan	N28°18′54″	E99°45′12″	3872
L. nelumbifolia	PG090934	Bealock, Daxueshan, Yunnan	N28°35′46″	E99°50′09″	4173
L. nelumbifolia	PG100816	Zhegushan, Hongyuan, Sichuan	N31°52′40″	E102°40′14″	3984
L. nelumbifolia	PG100820	Li County, Sichuan	N32°19′31″	E102°27′09″	3694
L. nelumbifolia	PG100828	Hongyuan, Sichuan	N32°42′30″	E102°08′50″	3748
L. nelumbifolia	PG110812	Balangshan, Wenchuan, Sichuan	N30°54′27″	E102°53′60″	4300
L. nelumbifolia	PG110825	Ganzi, Sichuan	N31°36′56″	E100°12′48″	3930
L. nelumbifolia	PG110843	Queershan, Baiyu County, Sichuan	N31°24′50″	E99°56′02″	4330
L. purdomii	PG100833	Gemoxiang, Aba, Sichuan	N333°02′03″	E101°33′29″	3429
L. purdomii	PG100838	Jiuzhi, Qinghai	N33°24′25″	E101°25′39″	3708
L. purdomii	PG100843	Tangkexiang, Aba, Sichuan	N33°02′57″	E102°17′13″	3562
L. yunnanensis	PG090982	Laojun Mountain, Yulong, Yunnan	N26°37′55″	E99°43′28″	3845
L. yunnanensis	PG100963	Cangshan, Dali, Yunnan	N25°40′45″	E100°05′36″	3780
L. atroviolacea	PG100907	Yancheng, Sichuan	N27°41′14″	E101°13′24″	3250
L. atroviolacea	PG100951	Wenhai, Lijiang, Yunnan	N26°58′44″	E100°10′25″	3120
L. cymbulifera	PG090910	Haba Snow Mountain, Yunnan	N27°40′07″	E99°48′32″	3888
L. cymbulifera	PG090915	Gezaxiang, Shangri-La, Yunnan	N28°02′52″	E99°45′56″	3164
L. cymbulifera	PG090936	Dongwang, Shangri-La, Yunnan	N28°36′55″	E99°49′19″	3749
L. cymbulifera	PG090937	Wumingshan, Xiangcheng, Sichuan	N29°07′20″	E100°00′54″	4130
L. cymbulifera	PG090950	Litang, Sichuan	N29°47′51″	E100°01′22″	—
L. cymbulifera	PG090962	Daocheng, Sichuan	N29°07′43″	E100°10′59″	3848
L. cymbulifera	PG090975	Baimaxueshan, Deqin, Yunnan	N28°24′01″	E98°58′58″	4141
L. lapathifolia	PG100920	Muli, Sichuan	N28°07′01″	E101°05′57″	3130
L. lapathifolia	PG100922	Wachang Street, Muli, Sichuan	N28°06′19″	E100°49′05″	2580

Table 1 continued

Species name	Sample ID	Locality	Latitude	Longitude	Atitude/m
L. lapathifolia	PG100942	Suberb, Lijiang, Yunnan	—	—	—
L. tongolensis	PG090906	Haba Snow Mountain, Yunnan	N27°38′53″	E99°47′53″	3717
L. tongolensis	PG090946	Chituxiang, Daocheng, Sichuan	N28°40′18″	E100°14′24″	3474
L. tongolensis	PG090949	Litang, Sichuan	N29°47′51″	E100°21′21″	3815
L. tongolensis	PG090965	Yading, Daocheng, Sichuan	N28°30′41″	E100°20′51″	3985
L. vellerea	PG090911	Haba Snow Mountain, Yunnan	N27°39′29″	E99°47′56″	3805
L. vellerea	PG090914	Zagexiang, Shangri-La, Yunnan	N28°07′43″	E99°49′47″	3711
L. vellerea	PG090932	Daxueshan, Shangri-La, Yunnan	N28°34′17″	E99°49′49″	4131
L. sibirica	PG100812	Zhegushan, Hongyuan, Sichuan	N31°53′13″	E102°40′07″	3900
L. sibirica	PG100857	Songfan, Sichuan	N32°53′04″	E103°29′03″	3230
L. sibirica	PG110876	Tianchi, Shangri-La, Yunnan	—	—	3890
L. cyathiceps	PG090904	Xiaozhongdian, Shangri-La, Yunnan	N27°38′32″	E99°47′48″	3602
L. cyathiceps	PG110872	Tianchi, Shangri-La, Yunnan	—	—	3890
L. lamarum	PG100829	Hongyuan, Sichuan	N32°43′34″	E102°06′32″	3911
L. lamarum	PG100945	Xuesongcun, Lijiang, Yunnan	N27°02′19″	E100°12′08″	3140
L. lamarum	PG100962	Cangshan, Dali, Yunnan	N25°40′63″	E100°05′34″	3800
L. subspicata	PG090912	Haba Snow Mountain, Yunnan	N27°39′29″	E99°47′56″	3802
L. subspicata	PG090925	Xiaoxueshan, Shangri-La, Yunnan	N28°18′53″	E99°45′01″	3871
L. subspicata	PG090938	Wumingshan, Xiangcheng, Sichuan	N29°07′14″	E100°01′23″	4202
L. subspicata	PG090959	Haizishan, Daocheng, Sichuan	N29°20′13″	E100°06′01″	4352
L. subspicata	PG090972	Dashan, Xiangcheng, Sichuan	N29°07′53″	E99°36′48″	3762
L. subspicata	PG090981	Laojun Mountain, Yulong, Yunnan	N26°37′55″	E99°43′27″	3846
L. subspicata	PG100915	Changhaizi, Muli, Sichuan	N28°07′28″	E101°11′09″	3630
L. subspicata	PG100927	Wachang Street, Muli, Sichuan	N28°03′13″	E100°46′08″	3950
L. hookeri	PG090918	Gezaxiang, Shangri-La, Yunnan	N28°07′59″	E99°53′18″	4111
L. hookeri	PG090980	Laojun Mountain, Yulong, Yunnan	N26°38′22″	E99°43′48″	3847
L. hookeri	PG100960	Cangshan, Dali, Yunnan	N25°40′48″	E100°05′41″	3760
L. fischeri	PG100810	Zhegushan, Hongyuan, Sichuan	N31°48′25″	E102°41′25″	3241
L. fischeri	PG110802	Jiajinshan, Baoxing, Sichuan	N30°51′30″	E102°43′13″	2870
L. fischeri	PG110805	Jiajinshan, Baoxing, Sichuan	N30°49′48″	E102°42′36″	3320
L. fischeri	PG110811	Jiajinshan, Xiaojin County, Sichuan	N30°51′25″	E102°41′23″	3913
L. fischeri	PG110815	Balangshan, Xiaojin County, Sichuan	N30°58′06″	E102°52′19″	3650
L. veitchiana	PG090985	Laojun Mountain, Yulong, Yunnan	N26°38′31″	E99°53′01″	2378
L. veitchiana	PG100856	Songfan, Sichuan	N32°53′04″	E103°29′03″	3230
L. veitchiana	PG100882	Jiuzhaigou, Sichuan	N32°54′32″	E104°14′42″	2686
L. anoleuca	PG090917	Gezaxiang, Shangri-La, Yunnan	N28°07′49″	E99°51′11″	3870
L. anoleuca	PG100706	Cangshan, Dali, Yunnan	—	—	—
L. anoleuca	PG100872	Wen County, Gansu	N33°03′20″	E104°41′57″	2059
L. anoleuca	PG100879	Tielouxiang, Wen County, Gansu	N32°52′00″	E104°22′09″	1871
L. latihastata	PG090908	Haba Snow Mountain, Yunnan	N27°38′53″	E99°47′48″	3717
L. latihastata	PG100708	Sanbaxiang, Shangri-La, Yunnan	—	—	—
L. latihastata	PG100953	Wenhai, Lijiang, Yunnan	N26°58′44″	E100°10′25″	3125
L. caloxantha	PG090979	Laojun Mountain, Yulong, Yunnan	N26°39′41″	E99°46′48″	3090
L. caloxantha	PG100702	Tuguancun, Diqing, Yunnan	N27°22′59″	E99°57′11″	2990
L. villosa	PG090903	Baishuitai, Shangri-La, Yunnan	N27°30′26″	E100°02′22″	2517
L. villosa	PG100909	Yancheng, Sichuan	N27°41′14″	E101°13′24″	3250
L. villosa	PG100937	Muli, Sichuan	N27°38′34″	E100°40′56″	3100
L. villosa	PG100941	Lugu Lake, Ninglang, Yunnan	N27°35′58″	E100°48′54″	2760
L. przewalskii	PG100807	Zhegushan, Li County, Sichuan	N31°43′46″	E102°44′31″	2994
L. przewalskii	PG100808	Yaxiucun, Hongyuan, Sichuan	N31°56′46″	E102°38′04″	3227
L. przewalskii	PG100858	Chuanzhu Temple, Songfan, Sichuan	N32°49′58″	E103°33′44″	3086
L. przewalskii	PG100863	Ruoergai, Sichuan	N34°07′08″	E102°38′48″	3580
L. przewalskii	PG100867	Lintan, Gansu	N34°36′43″	E103°42′39″	2875
L. przewalskii	PG100881	Jiuzhaigou, Sichuan	N32°54′32″	E104°14′42″	2686
L. przewalskii	PG110801	Baoxing, Sichuan	N30°38′28″	E102°49′29″	1800
L. przewalskii	PG110816	Balangshan, Xiaojin, Sichuan	N30°58′29″	E102°51′59″	3620
L. lankongensis	PG090923	Gezaxiang, Shangri-La, Yunnan	N28°07′34″	E99°45′17″	3036
L. lankongensis	PG090977	Nixixiang, Shangri-La, Yunnan	N28°03′13″	E99°30′15″	3155

Table 1 continued

Species name	Sample ID	Locality	Latitude	Longitude	Atitude/m
L. lankongensis	PG100931	Wachang Street, Muli, Sichuan	N28°02′19″	E100°45′58″	3810
L. lankongensis	PG100955	Wenhai, Lijiang, Yunnan	N26°57′01″	E100°10′51″	3030
L. kanaitzensis var. *kanaitzensis*	PG100939	Lugu Lake, Ninglang, Yunnan	N27°41′37″	E100°45′08″	2700
L. kanaitzensis var. *kanaitzensis*	PG100957	Heqing, Yunnan	N26°31′56″	E100°02′51″	3080
L. kanaitzensis var. *kanaitzensis*	PG100964	Cangshan, Dali, Yunnan	N25°42′11″	E100°07′28″	2420
L. kanaitzensis var. *subnudicaulis*	PG090902	Shangri-La, Yunnan	N27°40′32″	E100°01′32″	3560
L. kanaitzensis var. *subnudicaulis*	PG090983	Laojun Mountain, Yulong, Yunnan	N26°38′31″	E99°46′03″	3487
L. kanaitzensis var. *subnudicaulis*	PG090986	Cangshan, Dali, Yunnan	—	—	2900
L. kanaitzensis var. *subnudicaulis*	PG100701	Lidiping, Weixi, Yunnan	N27°09′38″	E99°25′02″	3207
L. kanaitzensis var. *subnudicaulis*	PG100952	Xuesongcun, Lijiang, Yunnan	—	—	—
L. kanaitzensis var. *subnudicaulis*	PG110818	Danba County, Sichuan	N30°35′31″	E101°39′37″	3100
L. tsangchanensis	PG090907	Haba Snow Mountain, Yunnan	N27°38′53″	E99°47′53″	3717
L. tsangchanensis	PG100938	Maoniushan, Ninglang, Yunnan	N27°40′01″	E100°36′40″	3963
L. tsangchanensis	PG110871	Tianchi, Shangri-La, Yunnan	—	—	3890
L. botryodes	PG100814	Zhegushan, Hongyuan, Sichuan	N31°52′40″	E102°40′14″	3984
L. botryodes	PG110836	Ganzi County, Sichuan	N31°02′40″	E99°17′12″	3500
L. sagitta	PG090948	Litang, Sichuan	N29°47′51″	E100°21′02″	3815
L. sagitta	PG100826	Qiongxi Town, Hongyuan, Sichuan	N32°49′37″	E102°30′33″	3691
L. sagitta	PG100834	Gemoxiang, Aba, Sichuan	N33°03′20″	E101°32′11″	3460
L. sagitta	PG100844	Tangkexiang, Ruoergai, Sichuan	N33°24′42″	E102°32′45″	3556
L. sagitta	PG100849	Ruoergai, Sichuan	N33°04′35″	E103°21′23″	3752
L. sagitta	PG100864	Luqu County, Gansu	N34°31′33″	E102°23′23″	3363
L. sagitta	PG100866	Lintan, Gansu	N34°45′04″	E103°15′09″	3130
L. melanocephala	PG090916	Zagexiang, Shangri-La, Yunnan	N28°07′49″	E99°50′37″	3796
L. melanocephala	PG100925	Wachang Street, Muli, Sichuan	N28°03′12″	E100°46′08″	3950
L. dictyoneura	PG090922	Pachahai, Shangri-La, Yunnan	N27°58′03″	E99°43′15″	3164
L. dictyoneura	PG090947	Chituxiang, Daocheng, Sichuan	N28°38′34″	E100°14′55″	3398
L. dictyoneura	PG090968	Xiangcheng, Sichuan	N29°00′33″	E99°43′34″	3920
L. dictyoneura	PG100709	Sanbaxiang, Shangri-La, Yunnan	—	—	—
L. dictyoneura	PG100905	Gesalaxiang, Yanbian, Sichuan	N27°08′01″	E101°17′28″	2580
L. dictyoneura	PG100921	Keerxiang, Muli, Sichuan	N28°07′00″	E101°05′58″	2800
L. dictyoneura	PG100954	Wenhai, Lijiang, Yunnan	N26°58′10″	E100°10′53″	3120
L. brassicoides	PG090953	Litang, Sichuan	N29°50′30″	E100°20′58″	4020
L. brassicoides	PG100811	Zhegushan, Hongyuan, Sichuan	N31°53′13″	E102°40′07″	3900
L. brassicoides	PG100836	Aba, Sichuan	N33°10′47″	E101°27′53″	3644
L. brassicoides	PG100853	Songfan, Sichuan	N32°55′34″	E103°20′59″	3672
L. brassicoides	PG110813	Balangshan, Wenchuan, Sichuan	N30°55′33″	E102°53′26″	4280
L. brassicoides	PG110826	Ganzi, Sichuan	N31°36′56″	E100°12′48″	3930
L. lingiana	PG110821	Gedaliangzi, Daofu, Sichuan	N30°32′13″	E101°35′17″	3820
L. lingiana	PG110848	Xinlong County, Sichuan	N30°14′50″	E100°15′31″	4060
L. pleurocaulis	PG090940	Wumingshan, Xiangcheng, Sichuan	N29°07′14″	E100°01′23″	4201
L. pleurocaulis	PG090958	Haizishan, Daocheng, Sichuan	N29°21′16″	E100°07′12″	4400
L. pleurocaulis	PG100818	Hongyuan, Sichuan	N31°52′27″	E102°43′58″	3756
L. pleurocaulis	PG110804	Jiajinshan, Baoxing, Sichuan	N30°49′35″	E102°42′43″	3300
L. virgaurea	PG090939	Wumingshan, Xiangcheng, Sichuan	N29°07′14″	E100°01′23″	4201
L. virgaurea	PG090954	Litang, Sichuan	N29°50′30″	E100°20′58″	4020
L. virgaurea	PG090960	Wumingshan, Daocheng, Sichuan	N29°20′46″	E100°06′01″	4352
L. virgaurea	PG090966	Xiangcheng, Sichuan	N29°00′08″	E99°44′26″	4026
L. virgaurea	PG100819	Hongyuan, Sichuan	N32°17′12″	E102°29′27″	3609
L. virgaurea	PG100837	Jiuzhi, Qinghai	N33°24′25″	E101°25′39″	3708
L. virgaurea	PG100840	Aba, Sichuan	N33°05′16″	E102°02′44″	3554
L. virgaurea	PG100845	Tangkexiang, Ruoergai, Sichuan	N33°24′42″	E102°32′45″	3556
L. virgaurea	PG100865	Luqu County, Gansu	N34°31′33″	E102°23′23″	3363

Table 2 The primer informations used in this study

DNA region	Primer pairs	Primer sequence (5′-3′)	Source
ITS	ITS4 ITS5	TCC TCC GCT TAT TGA TAT GC GGA AGT AAA AGT CGT AAC AAG G	White *et al.*, 1990
*mat*K	1F-KIM 1R-KIM	AAT ATC CAA ATA CCA AAT CC ACC CAG TCC ATC TGG AAA TCT TGG TTC	Kim (unpublished)
*psb*A-*trn*H	*psb*AF *trn*H2	GTT ATG CAT GAA CGT AAT GCT C CGC GCA TGG TGG ATT CAC AAT CC	Sang *at al.*, 1997; Tate and Simpson, 2003
*rbc*L	1F 724R	ATG TCA CCA CAA ACA GAA AC TCG CAT GTA CCT GCA GTA GC	Fay *et al.*, 1997

1.3 Data analysis

The raw DNA sequences were assembled and edited with SeqMan (DNA Star package; DNA Star Inc., Madison, WI, USA), and then were aligned with BioEdit V. 7 (Hall, 1999) and adjusted manually. MEGA 5.0 (Tamura *et al.*, 2007) was used to search the variable sites and calculate the intra- and inter-specific genetic distance. The neighbour-joining (NJ) tree was constructed under the Kimura-2-parameter (K2P) distance model recommended for species-level barcoding analysis (Hebert *et al.*, 2003), and bootstrap values were calculated with 1000 replications in MEGA 5.0. It is generally believed that when all the individuals of a species get together into a monophyletic clade with support rate ≥50%, this species is identified successfully (Tripathi *et al.*, 2013).

2 Results

Using selected primer pair listed in Table 2, the four loci used in this study were all successfully amplified and sequenced in 144 samples, which showed high universality. The sequence length/variable sites (bp) of ITS, *mat*K, *psb*A-*trn*H and *rbc*L were 689/191, 938/23, 586/42 and 633/5, respectively. The distributions of pair-wise K2P genetic distances were shown in Figure 1, indicating that a weaker barcoding gap existed in ITS, while the three chloroplast segments lacked this kind of barcoding gap.

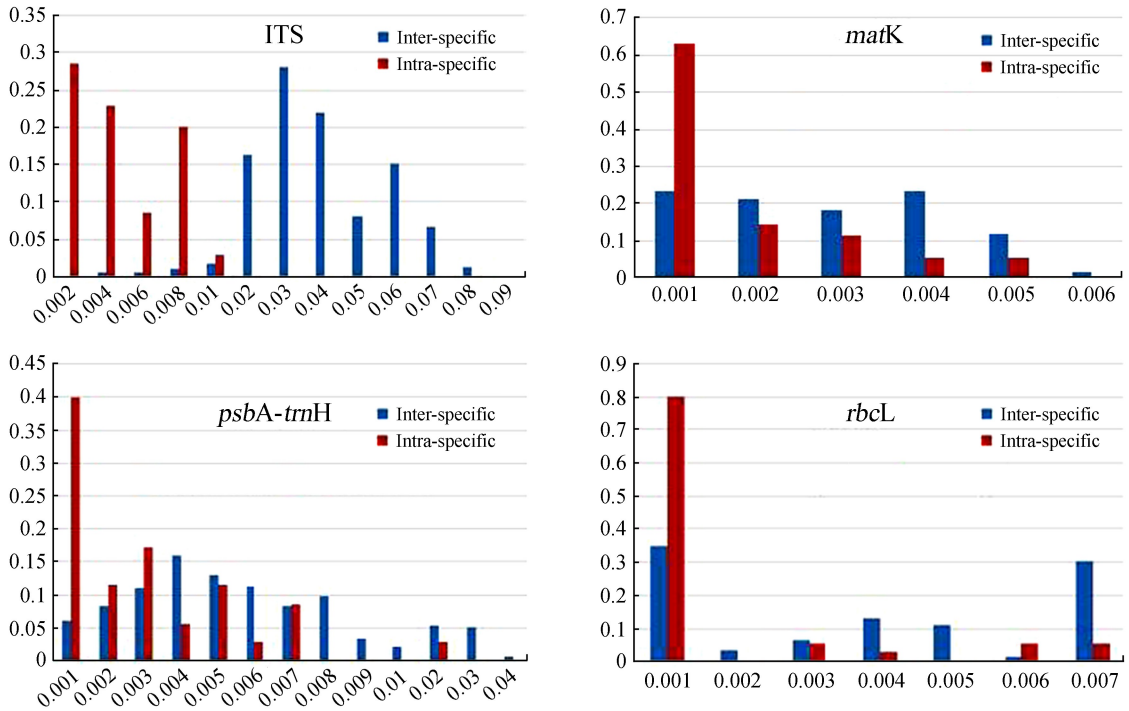

Fig. 1 Comparisons of frequency distribution of intra- and inter-specific pairwise distances among four core barcoding segments (x-axis: occurrence; y-axis: K2P distance)

The NJ trees (not shown) indicated that the three chloroplast sequences had extremely low species identification ability, of which *psb*A-*trn*H could identify one species (*L. lankongensis*) and the other two loci failed to identify any species. The forth loci, ITS, had the highest species discriminatory power for *Ligularia*. Of the 35 species analyzed, 21 were successfully identified using ITS with species identification rate reached 60% (Fig. 2). In addition, ITS sequence data alone or combined with *psb*A-*trn*H could separate Section *Corymbosae* from other Sections (Fig. 2, 3).

The analysis of any combination of the four regions showed that ITS + *rbc*L possessed the highest species discriminatory power with 21 species (60%) could be identified, which was the same as the resolving ability of single ITS region. Although the base mutation rates of *psb*A-*trn*H region was highest among the three cpDNA sequences (42/586), the combination of *psb*A-*trn*H + ITS could only discriminated 20 species (Fig. 3). The combination of all four regions also failed to improve the ability of species identification, and just 19 species were discriminated.

3 Discussion

An ideal barcode should possess sufficient variations among sequences of different species so as to discriminate species and should be sufficiently conserved in the sequences of the same species so that there is less variability intraspecific than interspecific (Kress *et al.*, 2005; Lahaye *et al.*, 2008; CBOL Plant Working Group, 2009). PCR and sequencing success rates are important criteria for DNA barcoding as well (Chase *et al.*, 2007; Kress and Erickson, 2007; Hollingsworth *et al.*, 2009). In the present research, four regions tested here showed a PCR and sequencing success rate of 100% in 144 individuals belonging to 35 species of *Ligularia*. which meaning that they had highly universal primer pair, and could generate high-quality bidirectional sequences. However, of the four core barcodes, only ITS generated a weak DNA barcoding gap and provided relatively the highest species resolution, and the three chloroplast regions did not exist any barcoding gap and had nearly no species discrimination power. This conclusion is consistent with that revealed by Gao *et al.* (2010) in the Asteraceae family.

*rbc*L variation mainly exists above the species level and the interspecific variation is usually low, while the evolution rates of *mat*K and *psb*A-*trn*H perform relatively fast in the chloroplast genome (Chase *et al.*, 1993; Shaw *et al.*, 2005). Previous studies on numerous land plants showed that single or combination of these three regions had high rate of species identification (Kress *et al.*, 2005, 2009; Kress and Erickson, 2007; Fazekas *et al.*, 2008; Lahaye *et al.*, 2008; Newmaster *et al.*, 2006, 2008; Hollingsworth *et al.*, 2009; Jiao and Shui, 2013; Liu *et al.*, 2013; Enan and Ahmed, 2014). However, the three chloroplast regions had lower nucleotide substitution rates than ITS and did not performed well in identifying *Ligularia*. For example, *psb*A-*trn*H could identify only one species, even if it was one of the chloroplast markers with fastest evolving rate and had high level of species discrimination in many plant groups. Furthermore, when ITS was combined with *psb*A-*trn*H, the species discriminatory power failed to improve. Congruence between the datasets of nuclear DNA marker (ITS) and chloroplast DNA markers (*mat*K, *rbc*L and *psb*A-*trn*H) were evaluated by the incongruence-length-difference (ILD) test showing that there was gene conflict between them. This kind of gene conflict probably is the cause for the decrease in species identification power of ITS when combined with *psb*A-*trn*H. Genus *Ligularia* was proposed to originated as a consequence of an explosive radiation within the last 20 million years (Liu *et al.*, 2006) and probably existed incomplete lineage sorting. Further, multiple hybridization and gene introgression occurred frequently among the congeneric species (Pan *et al.*, 2008; Yu *et al.*, 2011, 2014a, b). These phenomena could be the major causes of low species identification rate of DNA sequence. Anyway, we can't rely on a single

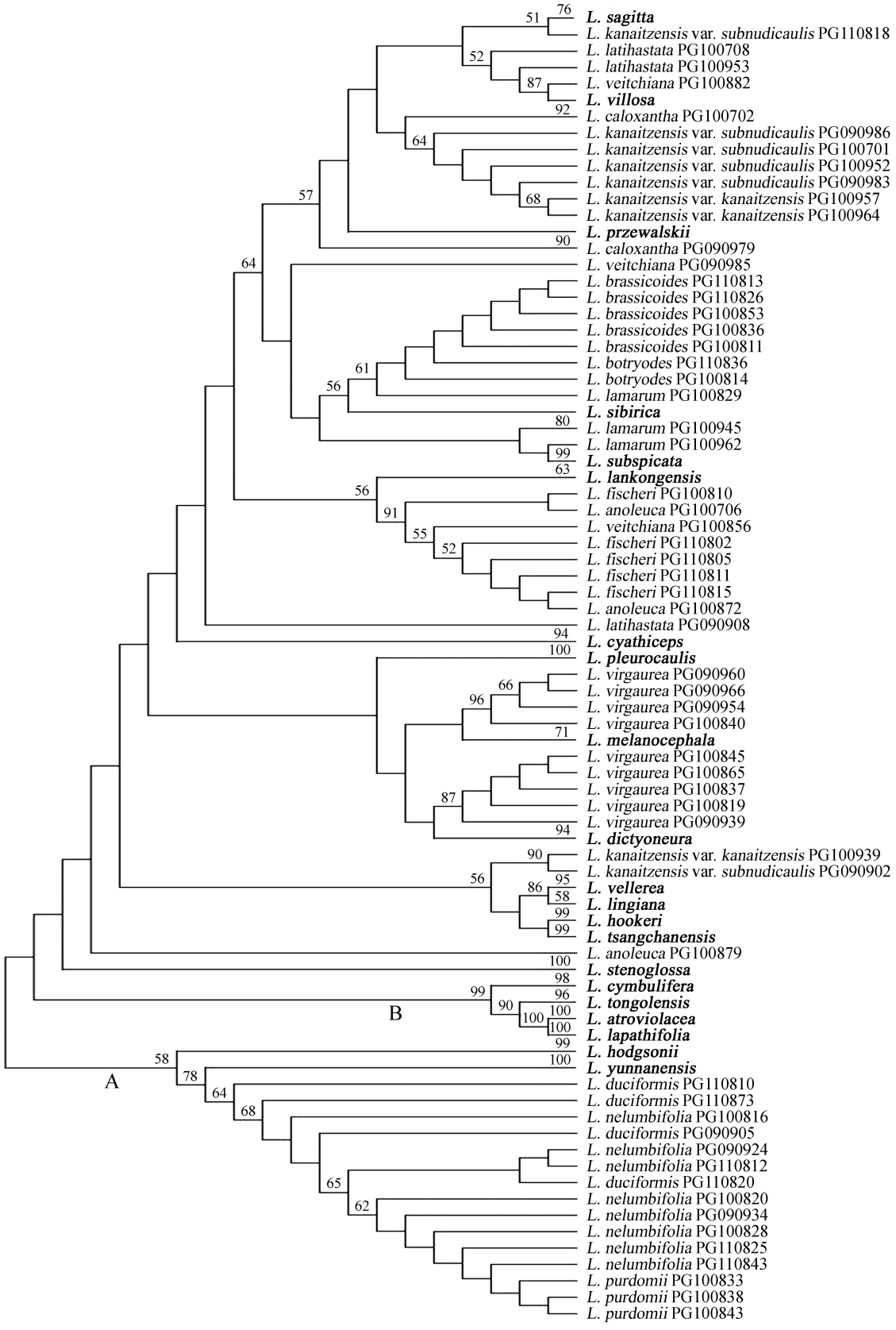

Fig. 2 The NJ tree inferring from the ITS sequence. Bootstrap values (>50%) are shown above the relevant branches. The blod black font shows species successfully identified. A: a clade with palmate veins; B: a clade with pinnate veins

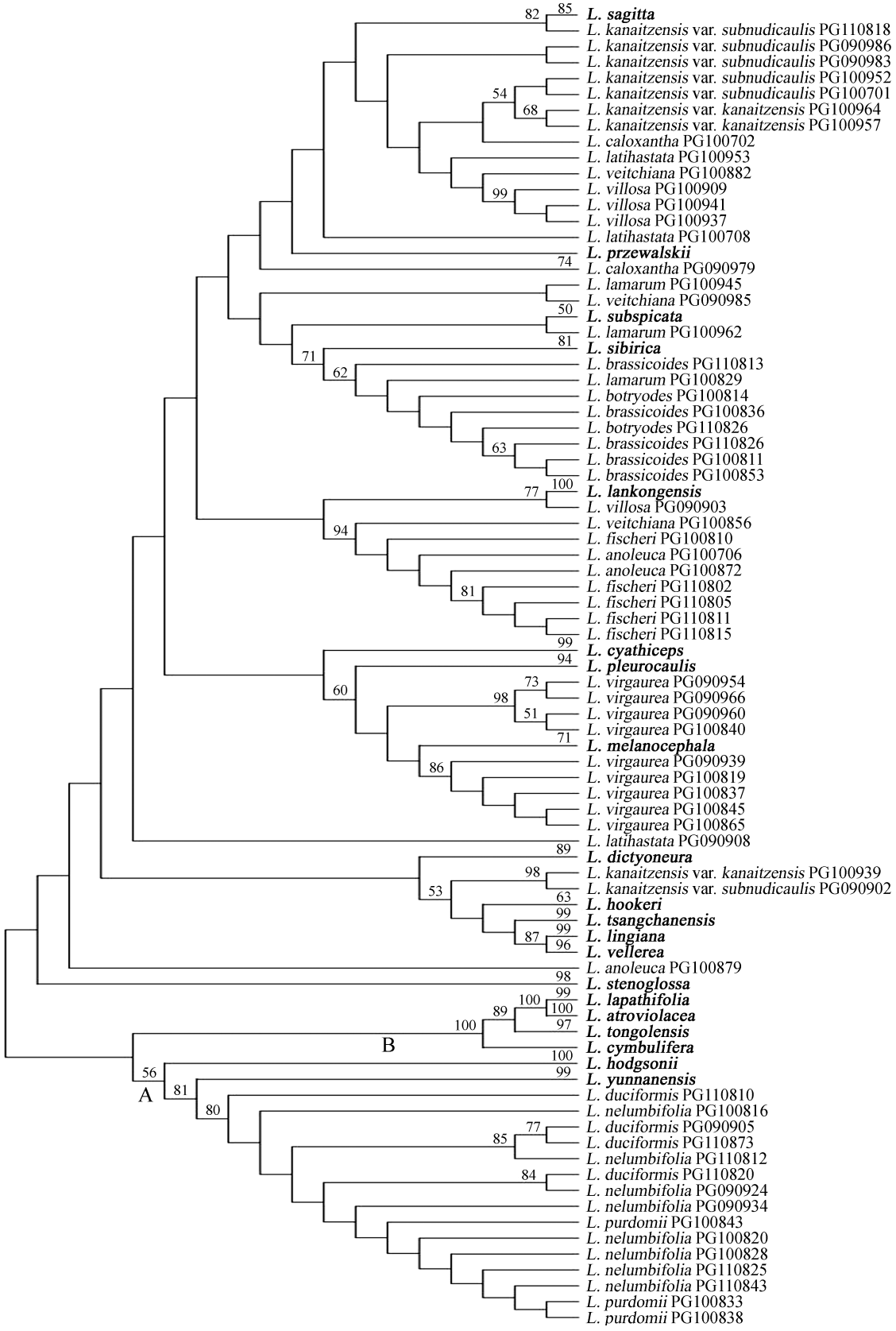

Fig. 3　The NJ tree inferring from the ITS+*psb*A-*trn*H sequence. Bootstrap values (>50%) are shown above the relevant branches. The blod black font shows species successfully identified. A: a clade with palmate veins; B: a clade with pinnate veins

nucleotide fragment (especially the uniparental inherited plastid markers) to provide reliable identification of hybrids from parental species (Newmaster *et al.*, 2006).

In *Ligularia*, section *Corymbosae* was considered as the most primitive group with the palmate or pinnate veins (Liu *et al.*, 1994). In the present study, NJ trees conduced from single ITS and combined ITS + *psb*A-*trn*H data showed that section *Corymbosae* was separated from the other sections of *Ligularia*, and the taxa with palmate veins (except *L. stenoglossa*) and ones with pinnate veins formed a clade, respectively. However, the DNA barcodes used in the present study could not identify other sections or series. Therefore, we need to search new chloroplast loci with faster evolution rate and higher interspecific variation, so as to be combined with ITS and serve as the "super-barcode" (Li *et al.*, 2015) for identifying *Ligularia* species.

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